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Potential sources of high value chemicals from leaves, stems and flowers of

Miscanthus sinensis ‘Goliath’ and *Miscanthus sacchariflorus*

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Abstract

Society demands chemicals from sustainable sources. Identification of commercially important chemicals in crops increases value in biorefineries and reduces reliance on petrochemicals. *Miscanthus sinensis* and *Miscanthus sacchariflorus* are high-yielding distinct plants, which are sources of high-value chemicals and bioethanol through fermentation. Cinnamates in leaves, stems and flowers were analysed by LC-ESI-MSⁿ.[†]

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[†]Abbreviations: ADME = absorption, disposition, metabolism, excretion; CADPE = 2-(3,4-dihydroxyphenyl)ethyl caffeate; CAPE = 2-phenylethyl caffeate; 2-CaffHydCitA = 2-caffeoylhydroxycitric acid; 3-*p*-CoQA = 3-*para*-coumaroylquinic acid; 4-*p*-CoQA = 4-*para*-coumaroylquinic acid; 5-*p*-CoQA = 5-*para*-coumaroylquinic acid; CaffQA = caffeoylquinic acid; 1-CaffQA = 1-caffeoylquinic acid; 3-CaffQA = 3-caffeoylquinic acid; 4-CaffQA = 4-caffeoylquinic acid; 5-CaffQA = 5-caffeoylquinic acid; CaffSA = caffeoylshikimic acid; 3-CaffSA = 3-caffeoylshikimic acid; 4-CaffSA = 4-caffeoylshikimic acid; 5-CaffSA = 5-caffeoylshikimic acid; 2-CaffTA = 2-caffeoylthreonic acid; 2,3-diHydBA = 2,3-dihydroxybenzoic acid; 2,5-diHydBA = 2,5-dihydroxybenzoic acid; 3,4-diHydBA = 3,4-dihydroxybenzoic acid; 2,3-diHydBA = 2,3-dihydroxybenzoic acid hexoside; 3,4-diHydBAHex = 3,4-dihydroxybenzoic acid hexoside; 1,4-diCaffQA = 1,4-dicaffeoylquinic acid; 1,5-diCaffQA = 1,5-dicaffeoylquinic acid; 3,4-diCaffQA = 3,4-dicaffeoylquinic acid; 3,5-diCaffQA = 3,5-dicaffeoylquinic acid; FA = ferulic acid; 2-FHydCitA = 2-feruloyl hydroxycitric acid; 3-FQA = 3-feruloylquinic acid; 4-FQA = 4-feruloylquinic acid; 5-FQA = 5-feruloylquinic acid; 2-FTA = 2-O-feruloylthreonic acid; FW = fresh weight; 2-HydBA = 2-hydroxybenzoic acid; 3-HydBA = 3-hydroxybenzoic acid; 4-HydBA = 4-hydroxybenzoic acid; HydBAHex = hydroxybenzoic acid hexoside; HIV-1 = human immunodeficiency virus-1; HPLC = high-performance liquid chromatography; LC-ESI-

Free phenols were extracted and separated chromatographically. More than twenty hydroxycinnamates were identified by UV and LC-ESI-MSⁿ. Several cinnamate hexosides were detected in the *M. sinensis* flower and in *M. sacchariflorus* (leaf and stem). Hydroxybenzoic acids and their hexosides were observed in leaf and stem of *M. sacchariflorus*. Higher concentrations of 3-feruloylquinic acid were observed in *M. sacchariflorus* stem, suggesting a role in cell-wall biosynthesis. This technique can be used to screen plants in a mapping family to identify genotypes / species with high concentrations of phenols. Plants with low concentrations of antimicrobial phenols may be good feedstocks for fermentation.

Highlights

- *Miscanthus sinensis* & *sacchariflorus*: sources of biofuel & platform chemicals
- LC-ESI-MSⁿ identified >20 hydroxycinnamates; flowers contained cinnamate hexosides
- Hydroxybenzoic acids and hexosides observed in leaf & stem of *M. sacchariflorus*
- More 3-FQA in *M. sacchariflorus* stem suggests a role in cell-wall biosynthesis
- Plants with low antimicrobial phenols may be good feedstocks for fermentation

Keywords: *Miscanthus sinensis*; *Miscanthus sacchariflorus*; hydroxybenzoic acids; hydroxycinnamate esters; high-value chemicals.

MS = liquid chromatography-electrospray ionisation mass spectrometry; *p*-CoA = *para*-coumaric acid; PDA = photodiode array; SA = syringic acid; UV = ultra-violet.

1. Introduction

Concerns about global warming and depletion of fossil fuels have stimulated interest in development of cleaner technologies that use sustainable and carbon-neutral feedstocks (Alonso et al., 2010; Sims et al., 2006). Sustainable feedstocks will be provided, at least in part, by the cultivation of non-food energy crops (Lewandowski et al., 2000; Sims et al., 2006). *Miscanthus* is a genus consisting of ca. fifteen species of perennial grasses that are native to tropical and subtropical regions of Africa, South Asia and temperate zones of Asia (Villaverde et al., 2010a, 2010b). *Miscanthus* × *giganteus* (*M.* × *giganteus*), a perennial rhizomatous grass with C₄-photosynthesis, can grow to heights of more than 3.5 m in a single growth season (Villaverde et al., 2010a, 2010b). This hybrid is a sterile triploid formed by a cross between *Miscanthus sacchariflorus* (*M. sacchariflorus*) and *Miscanthus sinensis* (*M. sinensis*). In this paper, we evaluate and compare the phenolic composition of these two claimed parent species of this hybrid.

There is much interest in using *Miscanthus* as an alternative to food crops and petroleum-based feedstocks to make a variety of bulk, intermediate and speciality chemicals, in addition to providing fuel. *Miscanthus* plants can grow under a range of climatic and environmental conditions (Lewandowski et al., 2000). After senescence, the plant can be burned for heat and electricity or fermented to produce biofuel. The chemical composition can affect the efficiency of conversion of biomass to energy and to chemical products (Klinke et al., 2004). Soluble phenols inhibit the bioconversion of sugars to ethanol, as many are toxic to the fermenting microorganisms (Klinke et al., 2004); thus it is important to profile the content of these unwanted components.

Plants produce a wide range of monophenolic and polyphenolic compounds with roles in strengthening cell walls, protection against UV radiation, tolerance to stress and resistance to pathogens. Hydroxycinnamic acids are the most widely distributed group of secondary compounds and are present as free, conjugated-soluble and bound-insoluble forms. They acids are often found as glycosides, sugar esters and amides. Caffeate esters predominate in grass, the most abundant being 5-O-caffeoylquinic acid. Hydroxycinnamic acids (specifically ferulic and *p*-coumaric acids) are covalently linked into the cell wall of grass species, where they cross link hemicellulose and lignin.

The potential benefits of these compounds are now appreciated by food and cosmetic industries (Dimitrios et al., 2006; Naczk and Shahidi, 2006, 2004; Ou et al., 2004; Padilla et al., 2005). Pharmaceutical companies continue to use natural products as sources of leads for the development of drugs (Harvey, 2008; Ou et al., 2004) and hydroxycinnamic acid conjugates are considered important drug leads (Touaibia et al., 2011). Many are reducing agents, hydrogen-donating antioxidants and quenchers of singlet oxygen (Parveen et al., 2011); thus diverse phenolic derivatives have interesting biological activities (Touaibia et al., 2011).

We recently reported the use of LC-ESI-MSⁿ to profile rapidly more than twenty phenols in the leaf and stem tissues of the hybrid *M. × giganteus*, including several novel mandelonitriles and mandelamides (Parveen et al., 2011). Total phenol concentration (hydroxycinnamates) in the leaf tissue was 0.04% FW and in the stem tissue 0.02% FW. The analysis was carried out on green material when the plant was at its highest biomass. Here we use LC-ESI-MSⁿ for qualitative and quantitative profiling of hydroxycinnamates in leaf, stem and flower extracts of the parental lines *M. sinensis*

and *M. sacchariflorus* at the same high biomass stage of growth as was *M. × giganteus*, to draw comparisons and conclusions about the relative utilities of these plants. Knowledge of the content and composition of the soluble phenols chemistry of these plants would help to determine the potential for exploiting this plant.

2. Results and discussion

2.1. Extraction of phenols

Soluble phenols were obtained from the methanol extract of fresh leaves, stems and flowers of *M. sinensis* and leaves and stems of *M. sacchariflorus*. More than twenty phenols (including hydroxycinnamic acid conjugates) were identified with UV absorptions typically in the region (240-340 nm) (Tables 2 and 3 (Supplementary Information) and Figures 1 and 2). The UV absorbance maxima could not be recorded for some cinnamic acid conjugates, either owing to low abundance or because they were masked by co-eluting peaks. The identification method used UV absorption coupled with the fragmentation pattern observed in tandem mass spectra using LC-ESI-MSⁿ. Where possible, the mass spectra and retention times of the phenols were compared with those of standards, including caffeic acid, *o*-coumaric acid, *p*-coumaric acid, cinnamic acid, ferulic acid, syringic acid, 5-caffeoylquinic acid (5-CaffQA, also known as chlorogenic acid), sinapic acid, 2-, 3-, and 4-hydroxybenzoic acid (2-HydBA, 3-HydBA and 4-HydBA), 2,3, 2,5- and 3,4-dihydroxybenzoic acid (2,3-diHydBA, 2,5-diHydBA and 3,4-diHydBA), 2-hydroxy-3-methoxybenzoic acid, 2-hydroxy-4-methoxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid (vanillic acid). In the absence of commercial standards, caffeoyl-, *p*-coumaroyl- and feruloyl- conjugates

were assigned primarily by their parent ion; their UV spectrum and elution order and assignments were supported by comparison of their mass spectrometric fragmentation data (MS^2 and MS^3) to those previously reported (Clifford et al., 2005; Jaiswal et al., 2010; Parveen et al., 2008;). In the absence of standards, all other identifications are considered provisional.

2.2. Characterisation of caffeoyl derivatives

This is the first study on the HPLC profiles of phenols in *M. sinensis* and *M. sacchariflorus* tissues. In all tissues, the most abundant compound was 5-O-caffeoylquinic acid (5-CaffQA). Particularly high concentrations of 3-O-caffeoylquinic acid (3-CaffQA) were detected in *M. sacchariflorus* tissues (Table 1 and Tables 2 and 3 (Supplementary Information)). The pattern of fragmentation and retention time for 5-CaffQA were consistent with those of a commercial standard. Diagnostic fragmentation ions of caffeoylquinic acids in negative-ion mode ESI- MS^n involved one of two pathways (i) loss of the acyl group with cleavage of the carbonyl-oxygen bond, (ii) β -elimination of a carboxylic acid (Parveen et al., 2011). Geometrical isomers were evident; for example, *cis* / *trans* isomers of caffeic acid were found in all extracts, based on photoirradiation experiments resulting in photo-isomerisation (Parveen et al., 2011). As observed previously in the stem extract of *M. sinensis*, a small but distinct peak showed properties very similar to that of 5-CaffQA in MS/MS negative-ion mode spectra. This was evidently a CaffQA -stereoisomer but 1-CaffQA was discounted, as this peak did not co-elute with an authentic sample of 1-CaffQA from acid-catalysed hydrolysis of cynarin (1,3-dicaffeoylquinic acid) (Parveen et al., 2011).

In *M. sinensis* leaf and *M. sacchariflorus* leaf and stem tissues, minor compounds giving peaks at m/z 335 $[M - H]^-$ and ions at m/z 317, 291, 179, 161, 135 were consistent with O-caffeoylshikimic acids (CaffSA) (Jaiswal et al., 2010). As for quinic acid, shikimic acid can form esters with cinnamic acids. Jaiswal et al. (2010) reported the synthesis and fragmentation pathways of 3-, 4-, and 5-CaffSA in negative ion mode.

CaffSAs have been widely reported in plants (Fang et al., 2002; Jaiswal et al., 2010); however, they were minor components in the *Miscanthus* tissue extracts. Three minor components with pseudomolecular ion 515 $(M - H)^-$ were detected in the leaf of *M. sacchariflorus* and were identified by their fragmentations patterns as 1,4-diCaffQA, 3,4-diCaffQA and 3,5-diCaffQA (Clifford et al., 2007). Dicafeoylquinic acids have previously been reported in *M. × giganteus* and in herbal chrysanthemum (Clifford et al., 2007; Parveen et al., 2011). Three further compounds in *M. sinensis* flowers and leaf and stem of *M. sacchariflorus* with pseudomolecular ion 515 $[M - H]^-$ were clearly hydroxycinnamates but not dicafeoylquinic acids. The MS² experiment yielded ions m/z 353, 341, 323, 191 and 179. These compounds were tentatively identified as caffeoylquinic acid hexosides where the hexose is linked to a hydroxy on a caffeoyl moiety as a glycoside; the linkage hexose-caffeoyl-quinic acid is demonstrated by the observation of fragment ions corresponding to CaffQAs. These compounds were previously reported in the hybrid *M. × giganteus* (Parveen et al., 2011). In *M. sacchariflorus*, compounds showing pseudomolecular ions and fragmentation patterns in negative-ion mode m/z 297 $[M - H]^-$ and 369 $[M - H]^-$ corresponded to 2-O-caffeoylthreonic acid (2-CaffTA) and 2-O- acid caffeoyloxycitric (2-CaffHydCitA), respectively. These compounds have previously been reported in *Dactylis glomerata* and in *Cornus controversa* (Lee et al., 2000; Parveen et al., 2011). Other caffeoyl-

derivatives were observed in *M. sacchariflorus* but these could not be identified.

Examples of structures are shown in Figure 3.

2.3. Characterisation of *p*-coumaroyl derivatives

The patterns of fragmentation observed at MS² and MS³ enabled the identification of three *trans*- isomers of O-*p*-coumaroylquinic acid (3-*p*-CoQA, 4-*p*-CoQA and 5-*p*-CoQA) in *M. sinensis* and *M. sacchariflorus* tissue extracts. The 5-acylated isomer was characterised by a MS² base ion m/z 191; 3-*p*-CoQA yielded MS² base peak m/z 163 [coumarate]⁻ and 4-*p*-CoQA was distinguished by β -elimination of coumaric acid to give MS² base ion m/z 173. Several isomers with *pseudomolecular* ions 325 [M - H]⁻, MS² base ion m/z 179 and secondary ions m/z 135, 281, 251 and 221 were identified as a *p*-coumaric acid hexoside in the flowers of *M. sinensis* and leaf and stem of *M. sacchariflorus* (Tables 2 and 3 (Supplementary Information)). Losses of 30, 60 and 90 are typical losses of CHOH units from sugars. Furthermore, in the flower extract, a minor distinct peak at t_R 9.3 min also gave a *pseudomolecular* ion m/z 341 [M - H]⁻ in the MS² spectrum. The UV absorption spectrum was not characteristic of O-acylquinates but rather of a cinnamic acid dimer (Pati et al., 2006). Thus we tentatively assign this compound as a caffeic acid dimer (Table 2). In both leaf and stem tissue of *M. sinensis*, a peak with a *pseudomolecular* ion m/z 389 [M - H]⁻ was detected in negative-ion mode MS. An MS² experiment yielded a major ion at m/z 371 and secondary ions at m/z 327, 297, 163 and 119. We can only speculate that this compound is a *p*-coumaroyl linked *via* an ester to a sugar unit comprising at least one carboxylic acid.

2.4. Characterisation of feruloyl derivatives

In *M. sinensis* and in *M. sacchariflorus* tissues, three *trans*-isomers of O-feruloylquinic acids (3-FQA, 4-FQA and 5-FQA) were readily identified by their *pseudomolecular* ion m/z 367 $[M - H]^-$; 3-FQA was distinguished by its MS^2 base ion m/z 193 [ferulate] $^-$, while 4-FQA was discriminated by the abundant peak m/z 173 $[M - \text{ferulic acid}]^-$ (from β -elimination) and the 5-isomer was characterised by the dominant fragment m/z 191 [quinate] $^-$ (Tables 2 and 3 (Supplementary Information)). Two geometrical isomers of 2-O-feruloyl-hydroxycitric acid (2-FHydCitA) were identified in the leaf and stem of *M. sacchariflorus* by their *pseudomolecular* ions $[383]^-$ and fragmentation ions 207, 193 and 189. This compound has been previously reported in *Dactylis glomerata* as a component of *Zea mays* (Ozawa et al., 1977; Parveen et al., 2008). In both plants, compounds with *pseudomolecular* ions 355 $[M - H]^-$, MS^2 ion m/z 193 and losses of 30, 60 and 90 corresponding to CHOH units were tentatively identified as ferulic acid hexosides.

2.5. Characterisation of other phenolic derivatives

Other minor components identified in *M. sacchariflorus* extract were a syringic acid hexoside identified by its *pseudomolecular* ion $[359]^-$ and MS^2 ions 197, 182 and 153 corresponding to the loss of a proton, a methyl group and a carboxylic acid (Tables 2 and 3 (Supplementary Information)). These compounds have previously been reported as constituents in dried plums (Fang et al., 2002). These compounds were only identified in *M. sacchariflorus* extracts. Standards were used for the identification of several hydroxybenzoic acids (HydBAs) and identified on the basis of MS data and LC retention times under conditions described above. These were 4-HydBA, 2,3-diHydBA,

3,4-diHydBA, vanillic acid and their sugar glycosides. These were detected in relatively high concentrations in leaves and stems of *M. sacchariflorus*.

2.6. Quantification and comparison of abundances

The abundances of selected compounds in the various extracts were by measurement of peak area in the HPLC chromatogram and reference to the extinction coefficient of chlorogenic acid. Data are presented in Table 1. Total hydroxycinnamates in *M. sinensis* were leaf = 1.2% FW; stem = 0.4% FW; flower = 0.7% FW and in *M. sacchariflorus* were leaf = 1.0% FW and stem = 1.1% FW.

Caffeoylquinic acids were the most abundant phenolic metabolites in all tissues examined; this was also true of the hybrid *M. × giganteus* (Parveen et al., 2011). However, there were marked differences in abundance of individual regioisomers between the species and between different tissues. For example, 3-CaffQA **1** was present to only 0.18-0.68 $\mu\text{mol g}^{-1}$ in *M. sinensis*, whereas it was more than an order of magnitude more abundant in *M. sacchariflorus* (7.0 $\mu\text{mol g}^{-1}$ in leaf and 4.5 $\mu\text{mol g}^{-1}$ in stem). It was also of low abundance in *M. × giganteus* (Parveen et al., 2011). By contrast, 4-CaffQA **14** was much more abundant in *M. sinensis* (12 $\mu\text{mol g}^{-1}$ in leaf, 3.0 $\mu\text{mol g}^{-1}$ in stem and 5.8 $\mu\text{mol g}^{-1}$ in flower) than it was in *M. sacchariflorus* (2.6 $\mu\text{mol g}^{-1}$ in leaf and not quantifiable in stem). 5-CaffQA **9** was present in high concentrations (3.3-13 $\mu\text{mol g}^{-1}$) in all tissues of *M. sinensis* and *M. sacchariflorus* examined. The total CaffQAs for the putative parental species were 28 $\mu\text{mol g}^{-1}$ (leaf) and 4.3 $\mu\text{mol g}^{-1}$ (stem) for *M. sinensis* and 23 $\mu\text{mol g}^{-1}$ (leaf) and 17 $\mu\text{mol g}^{-1}$ (stem) for *M.*

sacchariflorus, values which are much greater than those for the progeny *M. × giganteus* (1.0 $\mu\text{mol g}^{-1}$ and 0.1, respectively) (Parveen et al., 2011).

Coumaroylquinic acids **4,13,15,19,25** were present at much lower levels than were the CaffQAs in all tissues of *M. sinensis* and *M. sacchariflorus*; this is paralleled in the progeny *M. × giganteus* (Parveen et al., 2011). The picture is rather different for the feruloylquinic acids. Most of these are present in relatively low abundance in *M. sinensis* leaf (0.8-0.9 $\mu\text{mol g}^{-1}$), *M. sinensis* stem (0.5-1.1 $\mu\text{mol g}^{-1}$) and *M. sacchariflorus* leaf (0.2-1.3 $\mu\text{mol g}^{-1}$) but 3-FQA **11** was found at the remarkably high abundance of 7.7 $\mu\text{mol g}^{-1}$ in the stem of *M. sacchariflorus*. The total FQAs present in *M. × giganteus* stem were 3 nmol g^{-1} . Ferulic acid is known to be involved in biosynthesis of cell walls (Ralph et al., 1994) and these results may suggest that *M. sacchariflorus* is a more woody plant than are *M. sinensis* and *M. sacchariflorus*. *Miscanthus* species generally have a high lignin content and have been described as being closer in structure to a woody material than to a grass (Villaverde et al., 2010b, 2010c). Hydroxycinnamic acids, specifically ferulic acid and *para*-coumaric acid, are covalently linked into the cell wall of grass species, where they cross link hemicellulose and lignin by ester and ether bonds. It has been reported that their concentration in the cell wall is inversely correlated with recalcitrance of biomass to hydrolysis / saccharification (Akin, 2007). Microscopy of the stem tissue from *M. sinensis*, *M. sacchariflorus* and *M. × giganteus* correlated with the much higher amount of 3-FQA, in that the *M. sacchariflorus* stem had a considerably more woody structure. Figure 4 shows thin sections of the stems of *M. sinensis*, *M. sacchariflorus* and *M. × giganteus* stained with phloroglucinol to show cinnamic lignin. In *M. sinensis*, the lignin staining tends to be associated only with vascular bundles, whereas in *M. × giganteus* it is

somewhat more extensive, with some in the parenchyma. In *M. sacchariflorus*, almost all cells contain lignified precursors.

Caffeoylshikimic acids **20,21,24,26,68,75,76** were evident in *M. sinensis* leaf and *M. sacchariflorus* leaf and stem at low abundances; other acylshikimic acids were not detected. Small amounts of a wide range of hexosides were also observed.

Previously, we have shown that *M. × giganteus* stem extract contains a significant amount ($0.3 \mu\text{mol g}^{-1}$) of a mandelonitrile-CaffQA (Parveen et al., 2011). These were not detected in either of the two putative parent plants (*M. sinensis* and *M. sacchariflorus*). By contrast, we were unable to detect hydroxybenzoic acid derivatives in *M. × giganteus* but a number of derivatives were present in the two parental species in modest abundances (up to $1.3 \mu\text{mol g}^{-1}$ for **40** in *M. sacchariflorus* stem).

Hydroxybenzaldehyde was observed as a major component in the stem extract of *M. × giganteus* but was absent from the parental species. Syringic acid hexoside was also observed in *M. sacchariflorus* extracts. Could some of these compounds be markers for these plant species?

2.7. Applications

Phenols synthesised by plants are the most widespread antioxidants and 5-CaffQA is reported to accumulate to high concentrations in grasses such as *Miscanthus* (Parveen et al. 2008, 2011). Desirable properties of 5-CaffQA in the pharmaceutical and food industries include antioxidant, anti-carcinogenic, anti-inflammatory and cholesterol-lowering activities (Parveen et al., 2011). In addition, 5-CaffQA has been reported to protect against degenerative age-related diseases in animals when offered diets rich in

this compound (Niggeweg et al., 2004). Tomatoes have also been engineered with increased amounts of 5-CaffQA, with the potential to improve human health (Niggeweg et al., 2004). Caffeoylquinic acids are abundant in a wide range of fruit and vegetables and it has been claimed that up to 80% of cardiovascular disease, 90% of type II diabetes and one third of all cancers could be avoided by changes in lifestyle, in particular, changes to diet (Fraga, 2009). There is much epidemiological evidence that a diet rich in phenols can reduce incidences of such non-communicable diseases (Crozier et al., 2009). Caffeoylquinic acids have also been reported recently to protect against the aggregation and neurotoxicity of the 42-residue amyloid β -protein (Miyamae et al., 2012), although some issues in ADME have yet to be resolved.

Caffeic acid has a potential role in functional foods or pharmaceuticals due to its antioxidant, anti-inflammatory and anti-cancer properties (Cambie and Ferguson 2003; Touaibia et al., 2011). Caffeoyl esters, such as 2-phenylethyl caffeate (CAPE) inhibit of HIV-1 integrase (Fesen et al., 1994). By contrast, 2-(3,4-dihydroxyphenyl)ethyl caffeate (CADPE) inhibits growth and formation of colonies by HGC27 (gastric cancer), H1299 (lung carcinoma), A549 (lung carcinoma), HCT116 (colon cancer), and U2OS (osteosarcoma) human cancer cells through inducing senescence by increasing cellular size and cytoplasmic granularity. CADPE appeared to suppress significantly the expression of Twist 1, which is responsible for the up-regulation of p53, p21 and p16 proteins, in these lines (Dong et al., 2011). Both CAPE and CADPE have been isolated from *Sarcandra glabra* and *Teucrium pilosum* and have been used as an alternative to cytotoxic cancer therapy (Dong et al., 2011).

Large differences in abundances of some of the metabolites (as noted above) indicate that these might be developed as biomarkers for identification of *Miscanthus* species. The markedly different profiles of some metabolites also raise the question of whether *M. sinensis* and *M. sacchariflorus* are indeed the parents of *M. × giganteus*.

3. Conclusion

Profiles are reported of more than twenty hydroxycinnamates in *M. sinensis* and *M. sacchariflorus*, parental lines of *M. × giganteus*. Profile differences facilitate screening mapping populations, linking soluble chemotype populations with quality traits loci. This enables screening of plants in a mapping family to identify genotypes / species with high concentrations of valuable phenols. Plants with few antimicrobial phenols are good fermentation feedstocks. Knowledge of the phenolic profile in high biomass-yielding *Miscanthus* species can facilitate conversion into industrially useful products. Moreover, quinic acid is a key precursor of the anti-influenza drug oseltamavir (Tamiflu), suggesting *Miscanthus* as an alternative and reliable source. This early comparative study provides information on the content of potentially valuable compounds from readily renewable sources and possible biomarkers for identification.

4. Materials and methods

4.1. Plant materials

M. sinensis (cv. Goliath) and *M. sacchariflorus* were sown in experimental plots (20 m × 20 m) in 2009 at the Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth, UK. Bulk plant material was harvested (July 2009); leaves,

stems and flowers were separated, weighed and stored at -70°C for extraction and analysis.

4.2 Extraction and purification of phenols

Methanolic extracts were prepared as previously reported (Parveen et al., 2008).

Samples were analysed by HPLC (Figures 1 and 2).

4.3. Analytical HPLC

The Waters HPLC system (Waters Corporation, USA) included an auto-sampler and a 996 photodiode array (PDA) detector coupled to an analytical workstation. The column configuration consisted of a Waters C₁₈ reversed-phase Nova-Pak cartridge (4.0 µm, 8.0 mm × 100 mm). The sample injection volume was 25 µL.

The detection wavelength was set at 240–400 nm, the flow rate was 2.0 ml min⁻¹, the auto-sampler tray temperature was kept at 4°C and the column was at room temperature. The mobile phase comprised purified water-acetic acid (A; 95:5, v/v) and HPLC grade methanol (B). The initial condition was A:B (95:5, v/v) and the percentage of mobile phase B increased linearly to 100% over the 55 min run. Phenols were detected with a PDA detector at two wavelengths (λ_{max} 280 and 340 nm) and individual peaks were collected and solvents were evaporated under reduced pressure in a rotary evaporator. The software Empower (Waters Corporation, USA) was run on a Pentium III PC.

4.4. LC-ESI-MSⁿ analysis

The Thermo Finnigan HPLC/MSⁿ system (Thermo Electron Corporation, USA) included an on-line degasser, an auto-sampler, a column temperature controller, a photodiode array detector and a linear ion trap with ESI source, coupled to an analytical workstation. The configuration consisted of a Waters C₁₈ reversed-phase Nova-Pak column (4.0 µm, 3.9 mm × 100 mm). The sample injection volume was typically 10 µL.

The detection wavelength was set at 240-400 nm, the flow rate was 1.0 mL min⁻¹, with 100 µL min⁻¹ going to the mass spectrometer. The auto-sampler tray temperature was kept at 4°C and the column temperature was maintained at 30°C. The mobile phase consisted of purified water (A) and HPLC grade methanol-formic acid (B; 1000:1). The initial condition was A:B (95:5, v/v) and the percentage of mobile-phase B increased linearly to 100% over the 55 min run. The mass spectra were acquired. N₂ was used as the sheath and auxiliary gas and He was used as the collision gas. For the phenolic acids, the ionisation mode was negative and the interface and MSD parameters were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 15 units; spray voltage, 4.0 KV; capillary temperature 320°C; capillary voltage, 1 V; tube lens offset, 68 V.

4.5. Staining of stem sections

Free-hand sections of the stems were stained with 1% w/v phloroglucinol in aq. HCl (6.0 M). Sections were observed at ×10 magnification on a Leica DM6000B microscope with a Hitachi HV-D20 camera.

Supplementary Information

Table 2. HPLC / ESI-MS / MS (negative-ion mode) characterisation of hydroxycinnamates in extracts of *M. sinensis* leaf, stem and flower.

Table 3. HPLC / ESI-MS / MS (negative-ion mode) characterisation of hydroxycinnamates in extracts of *M. sacchariflorus* leaf and stem.

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Captions for Figures

Figure 1. HPLC/UV (340 nm) chromatograms of phenolic components in extracts of *Miscanthus sinensis* leaf, stem and flower. **1**, 3-CaffQA (*trans*); **2**, *p*-coumaric acid hexoside (*trans*); **3**, caffeic acid dimer (*trans*); **4**, 3-*p*-CoQA (*trans*); **5**, 4-CaffQA (*cis*); **6**, caffeic acid hexoside (*trans*); **7**, CaffQA stereoisomer (*trans*); **8**, 3-FQA (*cis*); **9**, 5-CaffQA (*trans*); **10**, caffeic acid hexoside (*trans*); **11**, 3-FQA (*trans*); **12**, ferulic acid hexoside (*trans*); **13**, 4-*p*-CoQA (*cis*); **14**, 4-CaffQA (*trans*); **15**, 5-*p*-CoQA (*trans*); **16**, *p*-coumaric acid hexoside (*trans*); **17**, 5-CaffQA (*cis*); **18**, 4-FQA (*cis*); **19**, 4-*p*-CoQA (*trans*); **20**, 5-CaffSA (*trans*); **21**, 3-CaffSA (*trans*); **22**, 5-FQA (*trans*); **23**, 4-FQA (*trans*); **24**, 5-CaffSA (*cis*); **25**, 5-*p*-CoQA (*cis*); **26**, 3-CaffSA (*cis*); **27**, 5-FQA (*cis*); **28**, *p*-coumaroyl ester.

Figure 2. HPLC/UV (340 nm) chromatograms of phenolic components in extracts of *Miscanthus sacchariflorus* leaf and stem. **29**, 4-hydroxybenzoic acid; **30**, 4-hydroxybenzoic acid hexoside; **31**, 3,4-dihydroxybenzoic acid hexoside; **32**, 3,4-dihydroxybenzoic acid hexoside; **33**, 2,5-dihydroxybenzoic acid; **34**, feruloyl-hydroxycitric acid; **35**, -3-CaffQA (*cis*); **36**, 3,4-dihydroxybenzoic acid hexoside; **37**, -4-hydroxybenzoic acid hexoside; **38**, 3-hydroxybenzoic acid; **39**, 3,4-dihydroxybenzoic acid hexoside; **40**, vanillic acid hexoside; **41**, feruloyl-hydroxycitric acid; **42**, 2-hydroxybenzoic acid; **1**, 3-CaffQA (*trans*); **33**, 4-CaffQA hexoside (*trans*); **44**, 2-CaffHydCitA (*trans*), **45**, 3,4-dihydroxybenzoic acid hexoside; **46**, vanillic acid hexoside; **47**, 2,3-dihydroxybenzoic acid; **48**, 3-CaffQA hexoside (*trans*); **49**, quinoil-*p*-coumaric acid (*trans*); **50**, 4-hydroxybenzoic acid hexoside; **51**, caffeic acid hexoside (*trans*); **52**, syringic acid hexoside (*trans*); **53**, *p*-coumaric acid hexoside (*trans*); **5**, 4-CaffQA (*cis*); **4**, 3-*p*-CoQA (*trans*); **54**, syringic acid (*trans*) hexoside; **55**, caffeic acid hexoside (*trans*); **56**, syringic acid hexoside (*trans*); **57**, vanillic acid; **58**, 1,5-diCaffQA (*trans*); **59**, *p*-coumaric acid hexoside; **8**, 3-FQA (*cis*); **60**, caffeic acid hexoside; **10**, caffeic acid hexoside (*trans*); **61**, unknown hexose / caffeic acid; **62**, ferulic acid (*trans*), **11**, 3-FQA (*trans*); **12**, ferulic acid hexoside (*trans*); **63**, unknown hexose / caffeic acid; **9**, 5-CaffQA (*trans*); **64**, caffeic acid hexoside; **65**, 3-CaffQA hexoside; **66**, isovanillic acid; **67**, caffeic acid hexoside; **14**, 4-CaffQA (*trans*); **68**, 4-CaffSA; **69**, *p*-coumaric acid; **70**, unknown caffeoyl compound; **71**, ferulic acid hexoside (*trans*); **72**, CaffQA stereoisomer; **73**, 2-CaffTA (*trans*); **18**, 4-FQA (*cis*); **19**, 4-*p*-CoQA (*trans*); **17**, 5-CaffQA (*cis*); **15**, 5-*p*-CoQA (*trans*); **74**, unknown hexose / caffeic acid; **75**, 4-CaffSA (*trans*) (*trans*); **23**, 4-FQA (*trans*); **25**, 5-*p*-CoQA (*cis*); **76**, CaffSA (*trans*); **22**, 5-FQA (*trans*); **77**, unknown hexose / caffeic acid; **78**, unknown hexose / caffeic acid; **26**, 3-

CaffSA (*cis*); **27**, 5-FQA (*cis*); **79**, CaffHydCitA (*trans*); **80**, unknown hexose / caffeic acid; **81**, *p*-CoSA; **82**, caffeic acid hexoside (*trans*); **83**, unknown hexose / caffeic acid; **84**, unknown hexose / caffeic acid; **85**, unknown hexose / caffeic acid; **86**, 3,4-diCaffQA (*trans*); **87**, 3,5-diCaffQA (*trans*); **88**, 1,4-diCaffQA (*trans*).

Figure 3. Exemplary structures of compounds.

Figure 4. Photomicrographs of phloroglucinol-stained sections of stems of *M. sinensis*, *M. sacchariflorus* and *M. × giganteus*.

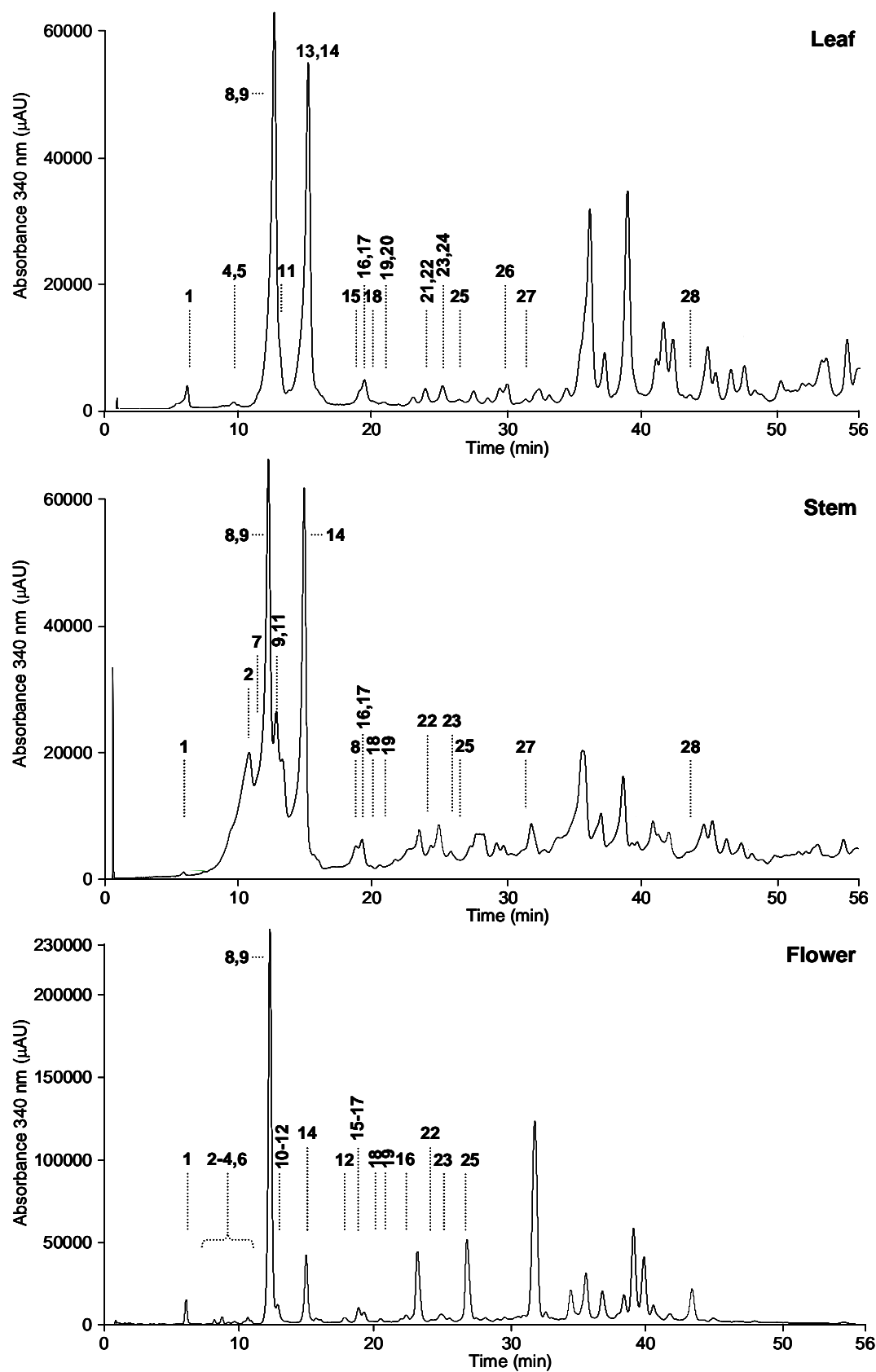


Figure 1.

Figure 2.

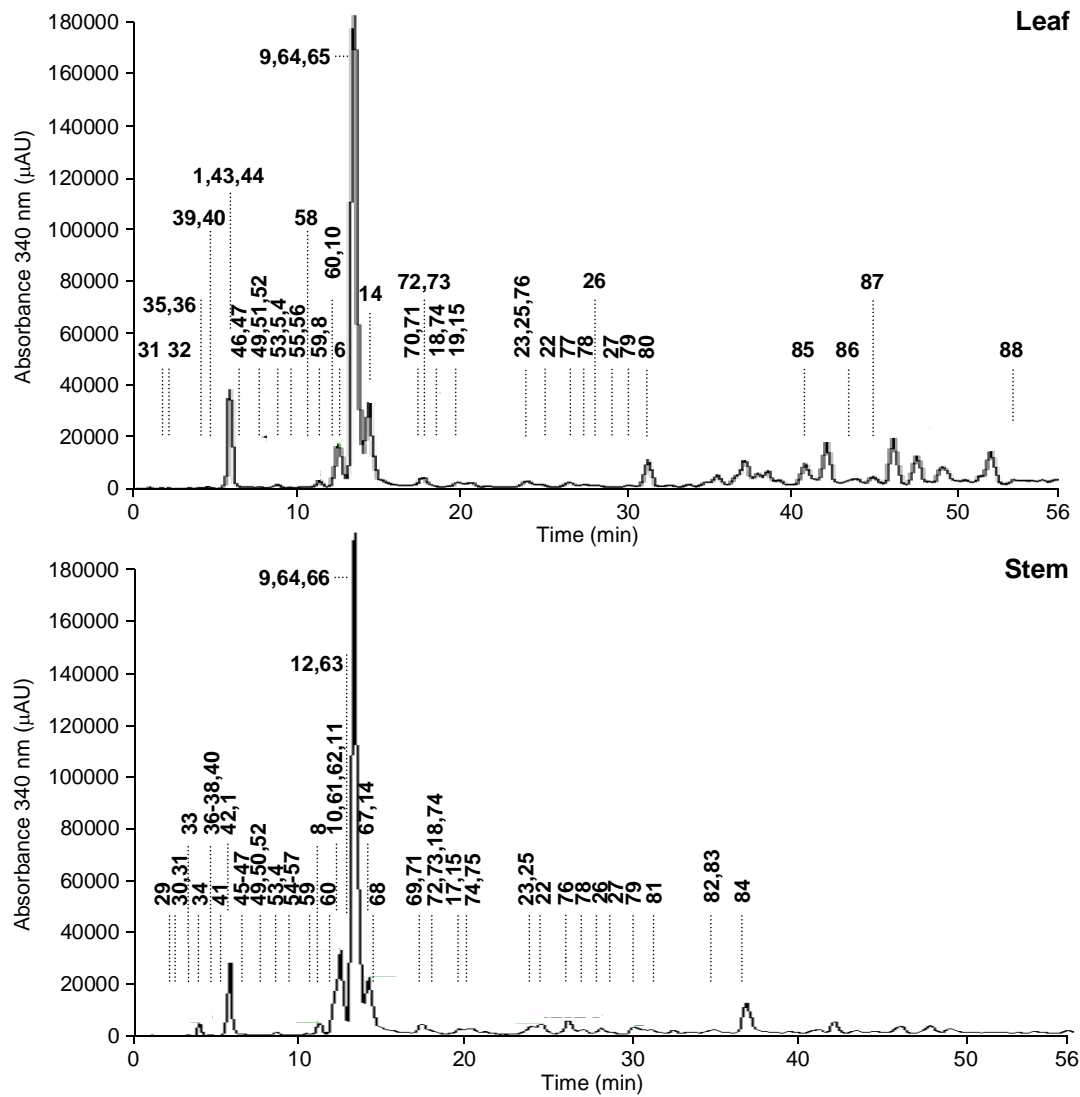


Figure 3.

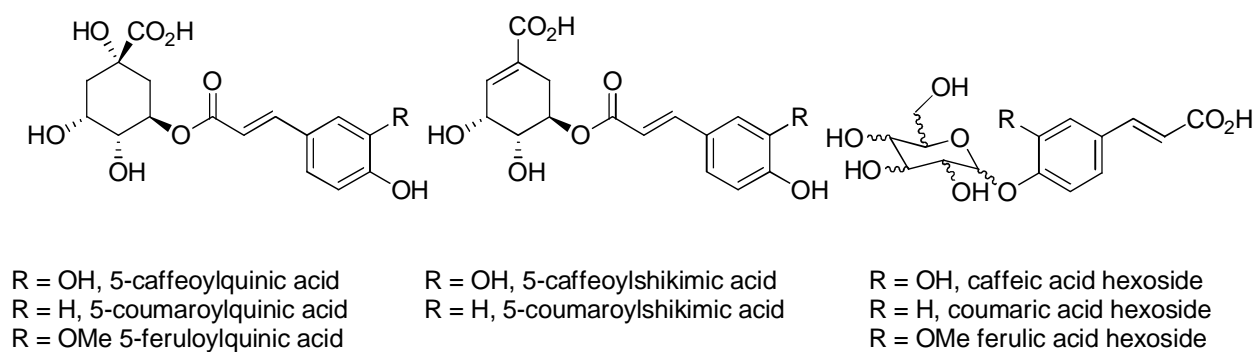


Figure 4.

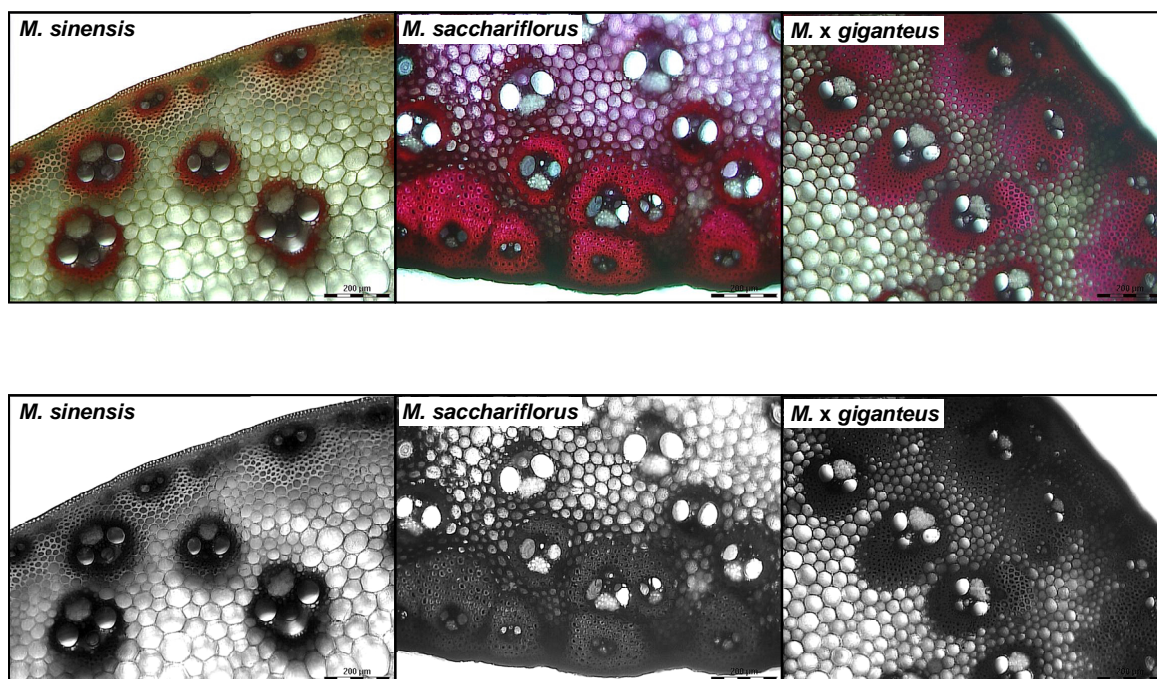


Table 1: Quantification of caffeoyl-, *p*-coumaroyl- and feruloyl- conjugates in the leaves, stems and flowers of *M. sinensis* and *M. sacchariflorus*.

Compound No.	Compound	<i>M. sinensis</i>			<i>M. sacchariflorus</i>	
		Leaf (µg/g fresh weight) (µmol g ⁻¹)	Stem (µg/g fresh weight) (µmol g ⁻¹)	Flower (µg/g fresh weight) (µmol g ⁻¹)	Leaf (µg/g fresh weight) (µmol g ⁻¹)	Stem (µg/g fresh weight) (µmol g ⁻¹)
<i>Caffeoylquinic acids (CaffQA)^a (M_r 354)</i>						
7	<i>CaffQA</i> stereoisomer (<i>t</i>)	-	62 (0.18)	- ^b	-	-
1	3-CaffQA (<i>t</i>)	239 (0.68)	62 (0.18)	120 (0.34)	2475 (7.0)	1595 (4.5)
35	3-CaffQA (<i>c</i>)	-	-	-	128 (0.36)	55 (0.16)
14	4-CaffQA (<i>t</i>)	4070 (12)	1053 (3.0)	2039 (5.8)	914 (2.6)	-
5	4-CaffQA (<i>c</i>) ^b	65 (0.18)	17 (0.050)	-	55 (0.16)	-
9	5-CaffQA (<i>t</i>)	4533 (13)	1173 (3.3)	2271 (6.4)	4002 (11.3)	4002 (11.3)
17	5-CaffQA (<i>c</i>)	788 (2.2)	204 (0.58)	395 (1.1)	518 (1.5)	221 (0.62)
<i>Caffeoylshikimic acids (CaffSA) (M_r 336)</i>						
21	3-CaffSA (<i>c</i>)	214 (0.64)	-	-	-	69 (0.21)
26	3-CaffSA (<i>t</i>)	17 (0.051)	-	-	189 (0.56)	-
75	4-CaffSA (<i>t</i>)	-	-	-	122 (0.36)	135 (0.40)
20	5-CaffSA (<i>t</i>)	114 (0.34)	-	-	-	-
24	5-CaffSA (<i>c</i>)	6.0 (0.018)	-	-	-	-
<i>Caffeic acid dimer (M_r 342)</i>						
3	Caffeic acid dimer	-	-	96 (0.28)	-	-
<i>Caffeoyl-hydroxycitric acid (CaffHydCitA) (M_r 370)</i>						
44	2-CaffHydCitA (<i>t</i>)	-	-	-	49 (0.13)	171 (0.46)
<i>Caffeic acid hexosides (CaffA hexoside) (M_r 342)</i>						
6	CaffA hexoside (<i>t</i> _R 9.9 min)	-	-	5 (0.015)	-	-

10	CaffA hexoside (t_R 12.8 min)	-	-	6 (0.018)	17 (0.05)	-
<u>Unknown (M_r 380)</u>						
61	caffeoyl (t_R 12.1 min)	-	-	-	-	527 (1.39)
78	caffeoyl (t_R 27.1 min)	-	-	-	98 (0.26)	96 (0.25)
<u>Unknown (M_r 412)</u>						
77	Caffeoyl	-	-	-	112 (0.27)	-
<u>p-Coumaroylquinic acids (p-CoQA) (M_r 338)</u>						
4	3-p-CoQA (t)	11 (0.033)	-	11 (0.033)	176 (0.52)	262 (0.78)
19	4-p-CoQA (t)	93 (0.28)	26 (0.077)	92 (0.27)	61 (0.18)	-
13	4-p-CoQA (c)	18 (0.053)	-	-	-	-
15	5-p-CoQA (t)	298 (0.88)	130 (0.38)	455 (1.35)	217 (0.64)	296 (0.88)
25	5-p-CoQA (c)	234 (0.69)	9.0 (0.027)	15 (0.044)	229 (0.68)	-
<u>p-Coumaric acid hexosides (p-CoA hexoside) (M_r 326)</u>						
2	p-CoA hexoside (t) (t_R 7.6 min)	-	-	406 (1.2)	-	-
53	p-CoA hexoside (t) (t_R 8.5 min)	-	-	-	-	74 (0.23)
16	p-CoA hexoside (t) (t_R 19.6 min)	-	-	770 (2.4)	-	-
<u>Feruloylquinic acids (FQA) (M_r 368)</u>						
11	3-FQA (t)	311 (0.85)	178 (0.48)	91 (0.25)	478 (1.3)	2822 (7.7)
8	3-FQA (c)	110 (0.30)	123 (0.33)	7 (0.02)	117 (0.32)	218 (0.59)
23	4-FQA (t)	328 (0.89)	182 (0.49)	106 (0.29)	78 (0.21)	13 (0.04)
18	4-FQA (c)	44 (0.12)	15 (0.04)	8 (0.02)	-	28 (0.08)
22	5-FQA (t)	332 (0.90)	425 (1.15)	75 (0.20)	94 (0.26)	341 (0.93)
27	5-FQA (c)	72 (0.20)	25 (0.07)	-	-	31 (0.08)
<u>Feruloyl-hydroxycitric acid (FHydCitA) (M_r 384)</u>						

34	2-FHydCitA (t) (t _R 3.9 min)	-	-	-	-	243 (0.63)
<u><i>Ferulic acid hexosides (FA hexoside) (M_r 356)</i></u>						
12	FA hexoside (t) (t _R 13.4 min)	-	-	21 (0.059)	-	-
71	FA hexoside (t) (t _R 17.1 min)	-	-	-	43 (0.12)	-
<u><i>Syringic acid hexosides (Hexoside-SA) (M_r 360)</i></u>						
52	SA hexoside (t) (t _R 7.9 min)	-	-	-	51 (0.14)	143 (0.40)
56	SA hexoside (t) (t _R 9.6 min)	-	-	-	22 (0.06)	137 (0.38)
<u><i>4-Hydroxybenzoic acid hexoside (M_r 300)</i></u>						
30	4-Hydroxybenzoic acid hexoside (t _R 2.9 min)	-	-	-	-	200 (0.67)
<u><i>3,4-Dihydroxybenzoic acid hexoside (M_r 316)</i></u>						
31	3,4-Dihydroxybenzoic acid hexoside (t _R 3.1 min)	-	-	-	196 (0.62)	237 (0.75)
36	3,4-Dihydroxybenzoic acid hexoside (t _R 4.7 min)	-	-	-	319 (1.0)	128 (0.41)
<u><i>Vanillic acid hexosides (M_r 330)</i></u>						
40	Vanillic acid hexoside (t _R 5.1 min)	-	-	-	60 (0.18)	432 (1.3)
46	Vanillic acid hexoside (t _R 6.2 min)	-	-	-	-	284 (0.86)

^a CaffQA = caffeoylquininic acid; CaffSA = caffeoylshikimic acid; CaffHydCitA = caffeoyl-hydroxycitric acid; CaffA = caffeic acid; CoQA = coumaroylquininic acid; CoA = coumaric acid; FQA = feruloylquininic acid; FHydCitA = feruloyl-hydroxycitric acid; FA = ferulic acid; SA = syringic acid.

^c- = not detected in tissue extract / not quantifiable